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USPT,JPAB,EPAB,DWPI,TDBD	l11 same l10	1	L12
USPT,JPAB,EPAB,DWPI,TDBD	flow cytometry	4328	L11
USPT,JPAB,EPAB,DWPI,TDBD	l7 same l8	76	L10
USPT,JPAB,EPAB,DWPI,TDBD	l7 and l8	233	L9
USPT,JPAB,EPAB,DWPI,TDBD	cryopreserv$7	1572	L8
USPT,JPAB,EPAB,DWPI,TDBD	sperm$5	15549	L7
USPT	(flow cytometry) and l3	20	L6
USPT	(flow cytometry) and l3	20	L5
USPT	flow cytometry and l3	20	L4
USPT	glycerol and l2	143	L3
USPT	centrifug$5 and l1	630	L2
USPT	((435/2)!.CCLS.)	952	L1

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L6: Entry 19 of 20 File: USPT Jun 4, 1991

DOCUMENT-IDENTIFIER: US 5021244 A
TITLE: Sex-associated membrane antibodies and their use for increasing the probability that offspring will be of a desired sex

BSPR:
Another potential separation approach for X-sperm and Y-sperm is based on the known differences in the DNA contents of X-sperm and Y-sperm. Because the DNA content of X-sperm cells is greater than the DNA content of Y-sperm cells, investigators hoped that the respective live cell populations could be separated by density gradient sedimentation or flow cytometry. However, neither has proven to be possible.

BSPR:
The present invention also provides for a method to separate living cells based on DNA content. The method involves separating the cells by flow cytometry, the cytometer having been improved to substantially increase its ability to recognize fine distinctions in fluorescence. Sperm cell populations separated in this manner result in subpopulations enriched in X- and Y-sperm. By isolating the membranes from these enriched subpopulations, one obtains another aspect of this invention: X- and Y-enriched sperm-plasma-membrane vesicles.

DEPR:
Immediately after collection we diluted freshly ejaculated mammalian semen with isoosmotic PBS to 15 ml, and slowly cooled it to 5.degree. C. At this temperature, we washed the sperm three times in isoosmotic, pH 7.2, tris-methylaminomethane buffered saline (TBS) saturated with phenylmethylsulfonylfluoride (PMSF) and 10 .mu.g/ml Hoechst No. 33342 bis benzimide dye. This solution stained cells and simultaneously inhibited enzymatic breakdown. We centrifuged the cells at 483.times.g for 20 minutes and resuspended them to remove seminal plasma proteins. We then diluted the washed cells in dye solution to a cell concentration of 20.times.10.sup.6 cells/ml and allowed them to stain for a minimum of 2 hours. (See Arndt-Jovin et al., 1977.) The Hoechst dye is available from Calbiochem-Behring Corp., San Diego, California. We prepared sheath fluid in the same manner as the solution used to wash sperm, except that the dye was omitted. We degassed the fluid as described above. The objective was to match the refractive indices between sheath and sample fluid.

DEPR:
We cavitated cell samples containing enriched X-sperm or Y-sperm subpopulations in Parr bombs. Suitable sample sizes were 3 to 10 ml with 50,000 to 500,000 cells/ml. We also cavitated non-enriched sperm samples (50:50 mixture of X- and Y-sperm). We used a cavitation method (at about 650 psi) as described by Gillis, et al., 1978. We separated plasma membrane vesicles consisting of mostly (e.g., 80% (boar sperm data)) head plasma membrane and some (e.g., 20% (boar sperm data)) tail plasma membrane from sperm heads, tails, and other particulates by pelleting centrifugation twice at 2500 x g for 30 minutes. We withdrew the supernatant containing the PMV material and centrifuged it at 100,000 x g to obtain the PMV material, which we resuspended and washed in 10 mM tris acetate (pH 5.5). This removed most of the TBS/PMSF/stain from the isolated PMV. Using this procedure we obtained three plasma membrane vesicles populations: (1) X-enriched sperm plasma

membrane vesicles (PMV-X) (approximately 68% X-sperm); (2) Y-enriched plasma membrane vesicles (PMV-Y) (approximately 72% Y-sperm); and (3) non-enriched plasma membrane vesicles (PMV-X/Y) (approximately equal amounts of X- and Y-sperm). The pelleted material resulting after centrifugation of X- and Y-enriched sperm subpopulations is the X- and Y-enriched non-membrane sperm component.

DEPR:
In a preferred 2-D gel separation process of this invention, we began with solubilized plasma membrane vesicles from whole sperm and from X- and Y-enriched sperm subpopulations isolated as in Example II. The LKB 2117 Multiphor II Electrophoresis System laboratory manual gives instructions and formulations for pouring 5.0% T, 2.7% C, 0.5 mm thick IPG gels with a broad range pH gradient of 4-10. We allowed gel polymerization to proceed for 1 hour in an oven heated to 50.degree. C. Following polymerization, we removed gels from the mold, washed them twice for 30 minutes in HPLC water and then rinsed them for 30 minutes in a solution of 2.5% glycerol. We air-dried the gels overnight in a dust free cabinet. Prior to focusing, we rehydrated IPG gels with a solution consisting of 8M urea, 10 mM dithiothreitol (DTT), 0.5% (volume/volume) nonidet p-40 (NP-40), 0.5% carrier ampholyte (CA). We utilized different brands of carrier ampholyte (LKB ampholine.RTM., Pharmalyte.RTM., and Servalyte.RTM. 3-10) to insure the best possible pH distribution. It is important that the carrier ampholyte used span the entire pI range over which the sample is to be focused. We included carrier ampholyte in rehydration of the gel in order to decrease hydrophobic interaction with basic immobilines.

DEPR:
We solubilized bull plasma membrane proteins with a solution containing 9M urea, 2% (weight/volume) DTT 2% (volume/volume) NP-40, 0.8% (volume/volume) carrier ampholyte. To aid in solubilization, we sonicated samples in a water bath sonicator at 4.degree. C. for 10 minutes. We pelleted remaining aggregates by centrifugation at 13,000.times.g for 10 minutes.

DEPR:
Following isoelectric focusing, we cut the gel into strips corresponding with sample wells. We incubated the strips in equilibration buffer and loaded them directly onto SDS slab gels. We equilibrated for 30 minutes with gentle shaking at room temperature in 8 ml of a solution containing 0.05M tris-HCl pH 6.8, 6M urea, 2% (weight/volume) SDS, 1% (weight/volume) DTT, 30% glycerol and 0.001% bromphenol blue. Following equilibration we rinsed the gel strips briefly to remove excess equilibration buffer and loaded them directly onto vertical 11.0% T, 2.7% C, 1.5 mm thick SDS slab gels with a stacker of 4.8% T, 2.7% C. We electrophoresed proteins at 100 volts until the dye front moved through the stacker. We then increased the voltage to 140 volts for the remainder of the run.

DEPR:
One day prior to fusion, myeloma cells were split to ensure that they were in log phase growth. On the day of fusion, the animal was sacrificed and splenectomized. We rinsed the spleen in sterile DMEM or RPMI-1640 (we prefer the latter) and teased it to separate splenocytes. (we now separate using syringe and needle perfusion.) Typical cell recovery from 1 spleen was 1.times.10.sup.8 cells. We washed splenocytes 3 times in DMEM or RPMI 1640. We prefer the latter.) The myeloma cells were also washed 3 times. We counted splenocytes and myeloma cells and mixed them together at a ratio of 7 spleen cells:1 myeloma cell. (We currently use 1:1.) We then centrifuged the cells at 1000 rpm (Mistral 3000) and decanted the supernatant.

DEPR:
Fusion was done with 1 ml of PEG for a period of one minute with gentle agitation. (We currently adjust PEG to pH 7.) We stopped the reaction with 20 ml DMEM (or preferably RPMI 1640) and centrifuged the mixture as before. We decanted the DMEM (or preferably RPMI 1640) and gently resuspended the pellet of fused cells in 12 ml of HAT (hypoxanthine aminopterin thymidine) medium and plated the cells into twenty-four wells (1 ml of cell suspension/well).

(We currently use HMT--hypoxanthine methotrexate thymidine.) We incubated plates overnight in a 7% CO.sub.2 incubator at 37.degree. C. The next day we fed the wells an additional ml of HAT (or HMT) medium and left them to incubate for 7 to 14 days. We removed the HAT (HMT) medium and replaced it with 10% FCS/DMEM or RPMI. (We prefer the latter.) We incubated plates until colony formation was visible. After the colonies had expanded into larger cultures, we froze them at -70.degree. C. and stored them long-term in liquid nitrogen.

DEPR:
We bound monoclonal antibodies to whole sperm by the following preferred method: We thawed antibody supernatants in a 37.degree. C. bath and diluted with a combination of Dulbecco's/PBS, Hoechst stain, and BSA as previously described. We diluted a whole sperm sample with a labeling solution to a final concentration of 480.times.10.sup.6 sperm cells per 12 mls. We added monoclonal antibody (ca. 200 .mu.l supernatant) to 1.times.10.sup.6 cells and allowed it to incubate for 1 hour at room temperature. We centrifuged the cells at 100.times.g for 5 minutes, discarded the supernatant and resuspended the cell pellet in 200 .mu.l of a 1:20 dilution of an affinity-purified anti-mouse antibody conjugated with R-Phycoerythrin. Following a second centrifugation, we discarded the supernatant and resuspended the cell mixture in a 10% neutral buffered formalin solution.

DEPC:
Sorting Sperm by Flow Cytometry

DEPU:
D. L. Garner et al., "Quantification Of The X-and Y-Chromosome-Bearing Spermatozoa of Domestic Animals By Flow Cytometry", Biol. Repro., 28, pp. 312-21 (1983) ("Garner-I")

DEPU:
J. M. Morrell et al., "Sexing of sperm by Flow Cytometry", Vet. Record., 122, pp. 322-324 (1988)

CCXR:
435/2

ORPL:
D. L. Garner et al., "Quantification Of The X-and Y-Chromosome-Bearing Spermatozoa of Domestic Animals By Flow Cytometry", Biol. Repro., 28, pp. 312-321 (1983) (Garner-I).

ORPL:
J. M. Morrell et al., "Sexing of Sperm by Flow Cytometry", Vet. Record., 122, pp. 322-324 (1988).

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L6: Entry 2 of 20 File: USPT Jun 6, 2000

DOCUMENT-IDENTIFIER: US 6071689 A
TITLE: System for improving yield of sexed embryos in mammals

BSPR:
This invention relates generally to the field of sex selection in mammalian offspring. It is especially relevant to the aspect of low dose artificial insemination for creating the desired sex of offspring. Particularly, the invention relates to systems for sorting sperm via flow cytometry for sex-specific and low dose efforts at artificial insemination or the like.

BSPR:
At present, the only quantitative technique used to achieve the separation of X-and Y-chromosome bearing sperm has been that involving individual discrimination and separation of the sperm through the techniques of flow cytometry. This technique appeared possible as a result of advances and discoveries involving the differential dye absorption of X-and Y- chromosome bearing sperm. This was discussed early in U.S. Pat. No. 4,362,246 and significantly expanded upon through the techniques disclosed by Lawrence Johnson in U.S. Pat. No. 5,135,759. The Johnson technique of utilizing flow cytometry to separate X- and Y- chromosome bearing sperm has been so significant an advancement that it has for the first time made the commercial separation of such sperm feasible. While still experimental, separation has been significantly enhanced through the utilization of high speed flow cytometers such as the MoFlo.RTM. flow cytometer produced by Cytomation, Inc. and discussed in a variety of other patents including U.S. Pat. Nos. 5,150,313, 5,602,039, 5,602,349, and 5,643,796 as well as international PCT patent publication WO 96/12171. While the utilization of Cytomation's MoFlo.RTM. cytometers has permitted great increases in speed, and while these speed increases are particularly relevant given the high number of sperm often used, certain problems have still remained. In spite of the almost ten-fold advances in speed possible by the MoFlo.RTM. flow cytometer, shorter and shorter sorting times have been desired for several reasons. First, it has been discovered that as a practical matter, the sperm are time-critical cells. They loose their effectiveness the longer they remain unused. Second, the collection, sorting, and insemination timings has made speed an item of high commercial importance. Thus, the time critical nature of the sperm cells and the process has made speed an essential element in achieving high efficacy and success rates.

BSPR:
In addition to the delicateness of the process, it has always been known that the sperm themselves are extremely delicate cells. While this factor at first glance seems like it might be considered easily understood, in fact, the full extent of the cells' sensitivities have not yet been fully explored. In the context of flow cytometry in general, most sorted cells or particles have often been spherical or otherwise physically able to withstand a variety of abuses. This is not the case for sperm cells. In fact, as the present invention discloses, the processing through normal flow cytometer techniques may, in fact, be unacceptable for cytometric sorting of sperm cells in certain applications. The sensitivities range from dilution problems and the flow cytometer's inherent need to isolate and distinguish each cell individually as well as the pressure and other stresses which typical flow cytometry has, prior to the present invention, imposed upon the cells or other substances

that it was sorting. This may also represent a unique factor for sperm cells because it appears that even though the sperm cell may appear to pass through the flow cytometer and be sorted with no visually discernable side-effects, in fact, the cells themselves may have been stressed to the point that they perform less than optimally in the insemination process. Thus, an interplay of factors seems involved and has raised unusual problems from the perspective of sperm cell sorting and ultimate use for artificial insemination.

BSPR:
Another problem which has been faced by those in the industry--again, in spite of the great advances by the Johnson patent and related technology--is the fact that the problem itself, namely, artificial insemination with a high success rate is one of a statistical nature in which a multitude of factors seem to interplay. Thus, the solutions proposed may to some degree involve a combination of factors which, when thoroughly statistically studied, will be shown to be necessary either in isolation or in combination with other factors. Such a determination is further compounded by the fact that the results themselves vary by species and may be difficult to ascertain due to the fact that testing and statistical sampling on a large enough data base is not likely to be worth the effort at the initial stages. For these reasons the invention can also involve a combination of factors which may, individually or in combination, represent the appropriate solutions for a given application. This disclosure is thus to be considered broad enough so that the various combinations and permeations of the techniques disclosed may be achieved. Undiscovered synergies may exist with other factors. Such factors may range from factors within the sorting or flow cytometer steps to those in the collection as well as insemination steps. At present, studies have been primarily achieved on bovine species, however, it is not believed that these techniques will be limited to such species or, for that matter to only sperm cells. It appears that the techniques used may have application beyond just sperm cells into areas which involve either sensitive items to be sorted or merely minimization of the impacts of the stresses of flow cytometry upon the item sorted.

DEPR:
As mentioned, the basic goal is that of separating the X-bearing sperm from the Y-bearing sperm. This is done in a manner which isolates the two types of sperm so that each can be separately packaged and dealt with. The isolation is preferably done through the use of flow cytometry. Flow cytometry in general is a technique which is well understood. For instance, the basic aspects of it are shown and discussed in a variety of patents to Cytomation, Inc. such as the U.S. Patents and other publications listed earlier. Each of these patents and the references cited therein, are incorporated by reference, thus those skilled in the art can easily understand the basic principles involved.

DEPR:
Essentially, flow cytometry involves sorting items, such as cells, which are provided to the flow cytometer instrument through some type of cell source. A conceptual instrument is shown in FIG. 1. The flow cytometer instrument includes a cell source (1) which acts to establish or supply cells or some other type of item to be analyzed by the flow cytometer. The cells are deposited within a nozzle (2) in a manner such that the cells are surrounded by a sheath fluid (3). The sheath fluid (3) is usually supplied by some sheath fluid source (4) so that as the cell source (1) supplies its cells, the sheath fluid (3) is concurrently fed through the nozzle (2). In this manner it can be easily understood how the sheath fluid (3) forms a sheath fluid environment for the cells. Since the various fluids are provided to the flow cytometer at some pressure, they flow out of nozzle (2) and exit at the nozzle orifice (5). By providing some type of oscillator (6) which may be very precisely controlled through an oscillator control (19), pressure waves may be established within the nozzle (2) and transmitted to the fluids exiting the nozzle (2) at nozzle orifice (5). Since the oscillator (6) thus acts upon the sheath fluid (3), the stream (7) exiting the nozzle orifice (5) eventually and regularly forms drops (8). Because the cells are surrounded by a sheath fluid environment, the drops (8) may contain within them individually isolated

(generally) cells or other items.

DEPR:
One of the aspects of flow cytometry which is particularly important to its application for sperm sorting is the high speed operation of a flow cytometer. Advances have been particularly made by the flow cytometers available through Cytomation, Inc. under the MoFlo.RTM. trademark. These flow cytometers have increased sorting speeds extraordinarily and have

DEPR:
thus made flow cytometry a technique which is likely to make feasible the commercial application of sperm sorting (among other commercial applications). They act to achieve high speed sorting, that is at a speed which is notably higher than those otherwise utilized. Specifically, Cytomation's MoFlo.RTM. flow cytometers act with oscillator frequencies of greater than about five kilohertz and more specifically can be operated in the 10 to 30 or even the 50 kilohertz ranges. Thus droplets are formed at very high frequencies and the cells contained within the sheath fluid environment can be emitted very rapidly from the nozzle (2). As a result, each of the components such as the nozzle (2) oscillator (6), and the like which make up and are part of a flow cytometer system result in a high speed cell sorter. In the application of a high speed cell sorter to the sorting of sperm cells, sorting at rates of greater than about 500 sorts per second is achieved. In fact, rates of sorting in the thousand and twelve hundred ranges have already been achieved through a high speed cell sorter. Importantly, it should be understood that the term "high speed" is a relative term such that as other advances in flow cytometry and specific applications are achieved, the aspect which is considered "high" may be varied or may remain absolute. In either definition, the general principle is that the sorting may occur at rates at which the parameters and physical characteristics of the flow cytometer are significant to the cells themselves when sorting particular cells such as sperm cells.

DEPR:
For the sheath fluid, a substance is selected according to one embodiment of the invention so that it may be chemically coordinated to prevent minimal changes. Thus, by selecting the appropriate sheath fluid not only in context of flow cytometry parameters, but rather also in context of the cell parameters themselves, the changes experienced by the cells and the over all result of the sorting can be enhanced. This is shown conceptually in FIG. 3. FIG. 3 shows some type of chemical factor (such as citrate or other factors) as it may exist throughout the various phases of the process. For instance, the four phases shown might represent the following: phase I may represent the existence of the cells within the cell source (1), phase II might show the existence of the cells as they are sorted in the sheath fluid environment, phase III might show the cells as they are collected after sorting and phase IV might show the reconstituted cells in a storage medium after sorting. These four phases as shown for the prior art may experience vastly different chemical factor environments. As shown conceptually, however, in the present invention the cells may experience very little change, most notably the dip or drop experienced between phases I and II may be virtually absent. This is as a result of the selection of the appropriate sheath fluid as mentioned above. Thus, as a result of being subjected to an appropriate sheath fluid, the cells in the present invention may experience a much lower level of stress.

DEPR:
The process of collecting, sorting, and eventually inseminating an animal through the use of flow cytometry involves a variety of steps. In the context of bovine insemination, first the semen is collected from the bull through the use of an artificial vagina. This occurs at rates of approximately 1.5 billion sperm per ml. This neat semen may be checked through the use of a spectrophotometer to assess concentration and may be microscopically evaluated to assure that it meets appropriate motility and viability standards. Antibiotics may then added. As a result the initial sample may have approximately 60 to 70 percent of the progressively motile sperm per ejaculate. For processing, a dilution through of some type TALP (tyrode

albumin lactate pyruvate) may be used to get the numbers of sperm at a manageable level (for flow analysis) of approximately 100 million per ml. The TALP not only nurtures the sperm cells, but it may make them hyper-activated for the staining step. Prior to staining, in some species such as the equine species, centrifugation may be accomplished. Staining may be accomplished according to a multi-stained or single-stained protocol, the latter, the subject of the Johnson Patent and related technology. The staining may be accomplished while also adjusting the extender to create the appropriate nutrient environment. In bovine applications this may involve adding approximately 20% egg yolk content in a citrate solution immediately after staining. Further, in staining the sperm cells, it has been discovered that by using higher amounts of stain than might to some extent be expected better results may be achieved. This high concentration staining may involve using amounts of stain in the tens of micro-molar content such as discussed in the examples below where 38 micro-molar content of Hoechst 33342 stain was used.

DEPR:
After adding the stain, an incubation period may be used such as incubating at one hour at 34.degree. C. to hasten the dye uptake with concentrations at about 100 million sperm cells per ml. Filtration may then be accomplished to remove clumps of sperm cells and then dilution or extending may or may not be accomplished to the desired sort concentration of approximately 100 million sperm cells per ml may be accomplished. Sorting according to the various techniques discussed earlier may then be accomplished from which sperm cells may be recovered in the collection phase. As mentioned earlier, the collection may result in samples with approximately 2% egg yolk citrate concentrate content (for bovine species). This sample may then be concentrated to about 3-5 million sperm cells per ml through the use of centrifugation after which the sheath fluid and preserving fluid may be removed. A final extension may then be accomplished with either 20% egg yolk citrate or a Cornell Universal Extender or the like. The Cornell Universal Extender may have the following composition for 1000 ml:

DEPR:
By example, twelve Angus crossbred heifers were superovulated using standard procedures: 6, 6, 4, 4, 2, 2, 2, and 2 mg FSH were injected intramuscularly at half-day intervals beginning between days 9 and 12 of the estrous cycle; 25 and 12.5 mg prostaglandin F-2 alpha were injected intramuscularly with the 6th and 7th FSH injections. Sperm from bulls of unknown fertility were stained with Hoechst 33342 and then sorted using a MoFlo.RTM. flow cytometer/cell sorter yielding 700-800 live sperm of each sex/sec. Average sort purity was 89% of the desired sex. Sorted sperm were concentrated to 3.36.times.10.sup.6 sperm/ml by centrifugation at 650 g for 10 min, cooled to 5.degree. C., and stored 4 h. Then 184 ul were loaded in 0.25 ml plastic straws; half the dose was inseminated into each uterine horn 20 to 24 h post-onset of estrus using automatic side-opening embryo transfer sheaths. Embryos were collected by standard non-surgical procedures at 7 or 16 days post-estrus. Results were similar between day 7 and 16 collections and between X- and Y-sorted sperm. Embryos were recovered from 9 heifers. There were 52 embryos (mean, 4.3.+-.5.3/donor) at normal stages of development, 13 retarded embryos and 31 unfertilized ova. Forty-six embryos were sexed by PCR using primers for a Y-chromosome-specific DNA sequence; 43 (93%) were of the intended sex. Although this study involved few animals, surprisingly, insemination of superovulated heifers with only 600,000 total (live) sexed unfrozen sperm gave similar results to conventional procedures. Variations on the above may also be accomplished, including, but not limited to, sorting through other than flow cytometric means, achieving superovulation in other manners, increasing fertility in other manners, and the like.

DEPR:
Angus heifers, 13-14 mo of age and in moderate body condition, were synchronized with 25 mg of prostaglandin F-2 alpha at 12-day intervals and inseminated 6-26 h after observed standing estrus. Freshly collected semen from three 14-26 mo old bulls was incubated in 38 .mu.M Hoechst 33342 at 75.times.10.sup.6 sperm/ml in a TALP medium for 1 h at 34.degree. C. Sperm

were sorted by sex chromosomes on the basis of epiflourescence from laser excitation at 351 and 364 nm at 150 mW using a MoFlo.RTM. flow cytometer/cell sorter operating at 50 psi and using 2.9% Na citrate as sheath fluid. X chromosome-bearing sperm (.about.90% purity as verified by resorting sonicated sperm aliquots) were collected at .about.500 live sperm/sec into 2-ml Eppendorf tubes containing 100 .mu.l Cornell Universal Extender (CUE) with 20% egg yolk. Collected sperm were centrifuged at 600.times.g for 10 min and resuspended to 1.63.times.10.sup.6 live sperm/ml in CUE. For a liquid semen unsexed control; Hoechst 33342-stained sperm were diluted with sheath fluid to 9.times.10.sup.5 sperm/ml and centrifuged and resuspended to 1.63.times.10.sup.6 progressively motile sperm/ml in CUE. Sexed semen and liquid control semen were cooled to 5.degree. C. over 75 min and loaded into 0.25-ml straws (184 ul/straw). Straws were transported at 3 to 5.degree. C. in a temperature-controlled beverage cooler 240 km for insemination 5 to 9 h after sorting. Sexed semen and liquid control semen were inseminated using side-opening blue sheaths (IMV), one half of each straw into each uterine horn (3.times.10.sup.5 live sperm/heifer). As a standard control, semen from the same bulls had been frozen in 0.5-cc straws by standard procedures (mean 15.6.times.10.sup.6 motile sperm/dose post-thaw), thawed at 35.degree. C. for 30 sec, and inseminated into the uterine body. Treatments were balanced over the 3 bulls and 2 inseminators in a ratio of 3:2:2 inseminations for the sexed semen and two controls. Pregnancy was determined ultrasonically 31-34 days after insemination and confirmed 64-67 days later when fetuses also were sexed (blindly). Data are presented in the table.

DEPR:
The objective was to determine pregnancy rates when heifers are inseminated with extremely low numbers of frozen sperm under ideal field conditions. Semen from three Holstein bulls of above average fertility was extended in homogenized milk, 7% glycerol (CSS) extender plus 5% homologous seminal plasma to 2.times.10.sup.5, 5.times.10.sup.5 or 10.times.10.sup.6 (control) total sperm per 0.25 ml French straw and frozen in moving liquid nitrogen vapor. Semen was thawed in 37.degree. C. water for 20 sec. Holstein heifers 13-15 mo of age weighing 350-450 kg were injected with 25 mg prostaglandin F-2-alpha (Lutalyse.RTM.) twice at a 12-day interval and inseminated with an embryo transfer straw gun and side-opening sheath, half of the semen deep into each uterine horn 12 or 24 h after detection of estrus. The experiment was done in five replicates over 5 months, and balanced over two insemination technicians. Ambient temperature at breeding was frequently -10 to -20.degree. C., so care was taken to keep insemination equipment warm. Pregnancy was determined by detection of a viable fetus using ultrasound 40-44 days post-estrus and confirmed 55-62 days post-estrus; 4 of 202 conceptuses were lost between these times. Day 55-62 pregnancy rates were 55/103 (53%), 71/101, (70%), and 72/102 (71%) for 2.times.10.sup.5, 5.times.10.sup.5 and 10.times.10.sup.6 total sperm/inseminate (P<0.1). Pregnancy rates were different (P<0.05) among bulls (59, 62, and 74%), but not between technicians (64 and 65%) or inseminations times post-estrus (65% for 12 h and 64% for 24 h, N=153 at each time). With the methods described, pregnancy rates in heifers were similar with 5.times.10.sup.5 and 10.times.10.sup.6 total sperm per inseminate.

DEPR:
Semen was collected from bulls at Atlantic Breeders Cooperative, diluted 1:4 with a HEPES-buffered extender +0.1% BSA, and transported 160 km (.about.2 HR) to Beltsville, Md. where it was sorted at ambient temperature by flow cytometry into a TEST yield (20%) extender using methods described previously (Biol Reprod 41:199). Sorting rates of up to 2.times.10.sup.6 sperm of each sex per 5-6 h at .about.90% purity were achieved. Sperm were concentrated by centrifugation (300 g for 4 min) to 2.times.10.sup.6 sperm/ml. Some sperm were sorted into extender containing homologous seminal plasma (final concentration, 5%). Sorted sperm were shipped by air to Colorado (.about.2,600 km) and stored at either ambient temperature or 5.degree. C. (cooled during shipping over 6 hr in an Equitainer, an insulated device with an ice-containing compartment). Heifers or dry cows detected in estrus 11 to 36 h earlier were inseminated within 9 to 29 h of the end of the sperm sorting session. Sperm (1 to 2.times.10.sup.5 in 0.1 ml) were deposited deep in the

uterine horn ipsilateral to the ovary with the largest follicle as determined by ultrasound at the time of insemination.

DEPR:
To summarize, it is possible to achieve pregnancies in cattle via artificial insemination of sperm sorted for sex chromosomes by flow cytometry, and the sex ratio of fetuses approximates that predicted by reanalysis of sorted sperm for DNA content (90%). However, pregnancy rates varied greatly in these preliminary experiments which required shipping sperm long distances. Fertility decreased drastically by 17 h post-sorting, but there was some confounding because different bulls were used at the different times. Further studies are needed to determine whether variation observed in pregnancy rates was due to bull differences, insemination techniques, interval between sorting and insemination, or other factors.

DETL:

I. FINAL COMPOSITION: 80% sodium citrate solution (72 mM) 20% (vol/vol) egg-yolk II. PREPARATION FOR 1 LITER: A. Sodium citrate solution 1. Place 29.0 grams of sodium citrate dihydrate (Na.sub.3 C.sub.6 H.sub.5 O.sub.7.2H.sub.2 O) in a 1,000 ml volumetric flask 2. Add deionized or Nanopure water to make 1,000 ml final volume. 3. Transfer to bottles and autoclave at 15 lbs pressure (245.degree. F.) for at least 30 minutes. a. Autoclave solution using conditions to minimize evaporation (loose cover) b. Be careful that water does not boil away. 4. Cool slowly at room temperature. 5. Store sealed in a 5.degree. C. cold room. B. Egg preparation 1. Obtain fresh hen's eggs from a good commercial source. 2. Wash the eggs free of dirt (do not use too much detergent) and rinse. 3. Immerse eggs in 70% ethanol for 2-5 minutes. 4. Remove eggs and allow to dry (or wipe dry) and store on a clean towel. C. Preparation of extender 1. Use sterile, clean glassware 2. A-fraction (non-glycerol fraction) a. Place 800 ml of 2.9% sodium citrate solution in a 1,000 ml graduated cylinder. b. Antibiotic levels for the non-glycerol containing fraction (A-fraction) of the extender may be as follows: I. Tylosin = 100 .mu.g/ml ii. Gentamicin = 500 .mu.g/ml iii. Linco-spectin = 300/600 .mu.g/ml c. Add 200 ml of fresh egg-yolk as outlined below (Section D) I. Mix very thoroughly. d. This provides A-fraction extender based on 2.9% sodium citrate, with 20% egg-yolk and antibiotics at concentrations known to be non-toxic to bull sperm. e. Extender can be stored overnight at 5.degree. C. f. Decant supernatant (upper 800 ml) the next day. g. Warm to 37.degree. C. prior to use the next day. D. To add egg-yolk to a buffered solution, the following procedure works well. 1. Wash egg and clean the eggs (see B above) 2. Open egg and separate yolk from albumin using a yolk separator. Alternatively, pour yolk back and forth 2-3 times between the two half shells. Do not rupture the membrane around the yolk. 3. Place the yolk onto a sterile piece of 15 cm filter paper. 4. Hold the filter paper over the graduated cylinder containing buffer and squeeze the yolk (rupturing the membrane) and allow the yolk to run out of the golded filter paper into the cylinder. Typically about 12-15 ml of the yolk can be obtained from one egg.

CCOR:
435/2

ORPL:
Rath, D., et al., "Production of Piglets Preselected for Sex Following in Vitro Fertilization with X and Y Chromosome-Bearing Spermatozoa Sorted by Flow Cytometry", Theriogenology, 47, 1997, pp 795-800.

ORPL:
Squires, E., "Simultaneous Analysis of Multiple Sperm Attributes by Flow Cytometry", Diagnostic Techniques and Assisted Reproductive Technology, The Veterinary Clinics of North America, Equine Practice, vol. 12, No. 1, Apr. 1996, pp 127-130.

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L6: Entry 11 of 20 File: USPT Nov 25, 1997

DOCUMENT-IDENTIFIER: US 5691133 A
TITLE: Method to quickly add cryoprotectants to sperm cells while maintaining viability

BSPV:
(1) the method is usable for other cryoprotectants besides the commonly used glycerol,

DEPR:
These equations respectively describe total membrane volume flux (J.sub.v) and transmembrane permeable solute flux (J.sub.CPA). Assuming the solution consists of a single permeable solute (here, CPA) and other impermeable solutes, the mathematical equations are: ##EQU1## where J.sub.v =total volume flux, V=sperm volume, t=time, N=mole number of solute, A.sub.c =sperm surface area, L.sub.p =water permeability coefficient of the sperm membrane, C=concentration of solute, J.sub.CPA =CPA flux across the cell membrane, superscript e=extracellular, superscript i=intracellular, C.sub.CPA =average CPA concentration of extracellular and intracellular concentrations. Furthermore, R=gas constant, T=absolute temperature, P.sub.CPA =CPA permeability coefficient of the sperm membrane, and .sigma.=the reflection coefficient of the particular CPA. The reflection coefficient is generally specific to a particular cryoprotectant and represents the opposing actions of water and cryoprotectant moving in opposite directions through the sperm cell membrane. Typically it is assumed to be 1. For example, using glycerol as the CPA, a value of 0.7 to 1 has only insignificant effect on the predictions found using this model. However, it must be remembered that its exact value is dependent on the cryoprotectant utilized and is a point where the user may fine tune the model for their particular use.

DEPR:
Examples of the physical values for human sperm needed are shown in Table 1. Similar values for other species of sperm are within the skill in the art to obtain. For example, the permeability coefficient of the cryoprotective agent can be determined using procedures as disclosed in Glycerol Permeability of Human Spermatozoa and its Activation Energy, by Gao, Mazur, Kleinhans, Watson, Noiles, and Critser, Cryobiology 29, 657-667, the disclosure of which is hereby incorporated by reference into this specification; or, the permeability coefficient of water can be determined using procedures as disclosed in Determination of Water Permeability Coefficient for Human Spermatozoa and its Activation Energy, by Noiles, Mazur, Watson, Kleinhans, and Critser; Biology of Reproduction 48, 99-109 (1993) the disclosure of which is also hereby incorporated by reference into this specification.

DEPR:
The human semen used in this example was obtained by masterbation from healthy donors after at least two days of sexual abstinence. The samples were allowed to liquefy in an incubator for 1 hour, at 37.degree. C., in high humidity, and in 5% CO.sub.2 and 95% air. A swim-up procedure was performed to separate motile sperm from immotile sperm. The motile sperm suspensions were centrifuged at 400.times.g for 7 minutes and then were resuspended in isotonic TL-Hepes medium, that is, HEPES-buffered TALP medium (286-290 mOsm) supplemented with Pyruvate (0.01 mg/ml) and BSA (4 mg/ml), at a cell concentration of 1.times.10.sup.9 sperm/ml.

DEPR:
A similar pattern, with the sperm's volume first shrinking or swelling and then recovering, takes place during the CPA addition or removal process from the sperm. Generally, when a cell is placed in a solution that is hyperosmotic with respect to the permeating solute (e.g. glycerol) but isotonic with respect to the impermeable salts, it first shrinks because of the osmotic efflux of intracellular water and then increases in volume as the solute (e.g. glycerol) permeates and as water concomitantly reenters the cell. When cells with CPA are exposed to an isotonic solution, they will swell because of osmotic influx of extracellular water and then decrease in volume as the CPA diffuses out of the sperm and as water concomitantly moves out.

DEPR:
The sperm's volume changes during a 1-step addition and a 1-step removal of 9.5-2M glycerol were calculated from computer simulation and are respectively shown in FIGS. 7 and 8. The higher the glycerol concentration, the longer the time period taken for the sperm volume recovery. However, typically it takes but a few seconds for human sperm to achieve their osmotic equilibration volumes. This means that the sperm experience the shrunken or swollen states for only an order of seconds during the addition or removal of glycerol. Therefore, the information concerning post-anisosmotic tolerance of the sperm returned to isotonic conditions after a short time exposure (an order of seconds) to the anisosmotic conditions is particularly important for designing optimal CPA addition/removal procedures to prevent sperm osmotic injury.

DEPR:
The kinetics of water and glycerol transport across the sperm membrane were modeled using standard computing techniques that are well within the skill of the art of this invention and using Equations 1 to 4. Two preferred schemes of CPA addition, Fixed-Volume-Step (FVS) and Fixed-Molarity-Step (FMS), were analyzed in this regard and are presented below.

DEPR:
Referring to FIG. 10, there is shown the calculated sperm volume change arising during a one-step, a two-step, and a four-step addition of glycerol to achieve a final 1M glycerol concentration at 22.degree. C. using an FMS addition.

DEPR:
Referring to FIG. 11, there is shown a comparison between a four-step FVS addition of glycerol and a four-step FMS of glycerol.

DEPR:
From FIGS. 9 to 11, a one-step addition of glycerol to sperm is predicted to cause a 10% to 20% sperm motility loss. This loss is predicted to occur because the minimum volume which the sperm can attain during this addition is approximately 72% of the original cell volume, a value less than the minimumly acceptable LVL of 75% previously determined above. In contrast, a four-step FVS or FMS addition of glycerol was predicted to greatly reduce such sperm motility loss (less than 5%).

DEPR:
Referring to FIG. 12, there is shown a one-step removal of glycerol. This removal was predicted to cause as high as 70% motility loss because the maximum cell volume during the glycerol removal was calculated to be over 1.6 times larger than the isotonic cell volume, much higher than the upper volume limit of the sperm.

DEPR:
Referring to FIG. 14, there is shown a comparison between the eight-step FMS and the eight-step FVS removal procedures. The eight-step FMS removal was predicted to prevent sperm motility loss over the FVS procedure. An eight-step FVS removal was predicted to cause a maximum cell swelling of over 1.2 times higher than isotonic cell volume while FMS removal was predicted to be much

lower than the UVL, indicating that the eight-step FVS removal is not as good as eight-step FMS removal. Also apparent from the computer simulation was the prediction that the human sperm would rapidly achieve an osmotic equilibrium (within seconds) during the 1-step or stepwise glycerol addition or removal. This further indicated that only a short time interval between steps of glycerol addition or removal was required.

DEPR:
In sum, a four-step FMS addition and an eight-step FMS removal of glycerol were predicted to be optimal protocols to prevent sperm motility loss in human sperm using the foregoing equations.

DEPR:
A two-step removal of CPA from the cells using a nonpermeating solute as an osmotic buffer has been previously used to avoid the osmotic injury to other cell-types. The detailed procedure is: (1) the CPA is directly removed from the cells by transferring cells to a hyperosmotic medium (osmotic buffer) containing no CPA but only nonpermeating solutes, and then (2) the cells are directly transferred to an isotonic solution. It has been known that 600 mOsm is the hyperosmotic upper tolerance limit for human sperm (FIG. 9). Therefore, osmolality of the osmotic buffer medium should not be over 600 mOsm. Under this limit, a hyperosmolality of 600 mOsm is expected to provide the maximum "buffer effect" to reduce the sperm volume swelling during the first step of the "two-step" procedure for glycerol removal. Sperm volume change during the 2-step glycerol (1M) removal process using a 600 mOsm buffer medium was calculated and shown in FIG. 15. It was predicted that maximum volume the sperm would achieve 1.25 times of the isotonic cell volume, which is higher than the UVL of the sperm, and might cause over 50% sperm motility loss.

DEPR:
TL-HEPES medium with 2M glycerol was either one-step or stepwise added to an equal volume of the isotonic sperm suspension to achieve a final 1M glycerol concentration at 22.degree. C. Glycerol in the sperm was removed by dilution either by a one-step or stepwise addition of the TL-HEPES medium with or without an osmotic buffer (sucrose) placed into the cell suspension. The detailed procedures for the glycerol addition and removal are described in Tables 2-5. Sperm motility before, during, and after the different glycerol addition and removal procedures was measured by CASA. The membrane integrity of the sperm was determined by the dual staining technique and flow cytometry.

DEPR:
Add 2000 .mu.l of isotonic solution directly to 100 .mu.l of cell suspension with 1M glycerol.

DEPR:
Glycerol was experimentally added to or removed from the human sperm using the stepwise procedures as predicted from the computer simulation. Percent motility of the human sperm after one step or a four-step FMS addition of glycerol (Table 2) is shown in FIG. 16. One step addition created approximately 20% sperm motility loss while the four-step addition, less than 8%. FIG. 17 shows effects of different glycerol removal procedures (Table 3) on the human sperm motility loss. Less than 30% motile sperm kept motility after one-step removal of glycerol while majority of sperm (over 92%) maintained the motility after the eight-step FMS removal. The motility loss caused by a two-step removal of glycerol using sucrose as a non-permeating buffer (total osmolality of the buffer medium was 600 mOsm) was close to 45%. Thirty-five percent of the sperm lost motility after a eight-step FVS removal of glycerol. The experimental results agree well with the predictions generated from computer simulation.

DEPR:
FIG. 18 shows the membrane integrity of the human sperm in 1M glycerol solution or after addition and removal of 1M glycerol by the different procedures. The sperm appeared to maintain membrane integrity under all

experimental conditions.

DEPR:
This example presented two CPA addition or removal schemes (FVS and FMS). As the example shows, the Fixed-Molarity-Step is preferable to reduce osmotic injury over the Fixed-Volume-Step. In particular, for human sperm, the example shows that a four-step FMS addition of glycerol to the sperm and an eight-step FMS removal of glycerol from the sperm were predicted to be optimal, which was confirmed upon implementation. Upon reviewing each scheme, the minimum/maximum cell volumes after each step of FVS addition/removal was uneven or unequal, some of which exceeded the LVL and UVL of the sperm. In contrast, minimum/maximum cell volumes after each step of FMS addition/removal of glycerol were shown to be relative even (FIGS. 11 and 14). For a fixed number of steps, the extent of cell volume change during CPA addition/removal using the FMS scheme is much smaller than that using the FVS scheme (also see FIGS. 11 and 14).

DEPL:
where M.sub.s =CPA concentration in sperm suspension (molarity) after n-1 step dilutions, M.sub.o =CPA concentration in initial sperm suspension (molarity), n=total number of steps, i=the ith step addition, V.sub.o =original volume of sperm suspension (ml) and V.sub.i =volume of isotonic solution added into the sperm suspension at the ith step. After n-1 steps of adding isotonic solution into the sperm suspension, the diluted sperm suspension is centrifuged, for example, at 400 g for 5-7 minutes., and then the sperm pellet is resuspended in isotonic solution to make the last (nth) step dilution.

DEPL:
Glycerol permeability coefficient(Ps) 1.1.times.10.sup.-3 cm/minutes (22.degree. C.)

DEPL:
Table 4. Procedures used in 2-step removal of 1M glycerol from the human sperm using sucrose as an osmotic buffer

DEPC:
Prediction of Optimal Conditions for Glycerol Addition or Removal

DEPV:
(1) Add 2000 .mu.l of sucrose buffer medium (TALP+sucrose, 600 mOsm) to 100 .mu.l of sperm suspension with 1M glycerol; (2) centrifuge the suspension (400 g for 7 minutes) and take off the supernatant; and (3) resuspend cell pellet with 500 ul isotonic TALP medium.

DETL:
TABLE 2 _____ Procedures used in 4-step addition of 1 ml of 2 M glycerol solution of 1 ml of isotonic sperm suspension FVS FMS _____ Add 0.25 ml of 2 M glycerol Stepwise add 0.14, 0.19, 0.27 4 times to 1 ml isotonic and 0.4 ml of 2 M glycerol sperm suspension to isotonic sperm suspension

_____

DETL:
TABLE 3 _____ Procedures used in 1-step and 8-step removal of 1 M glycerol from the human sperm 8-Step Dilution FVS FMS _____ Add 100 .mu.l of isotonic TALP Stepwise add 14.3, 19, 26.6, 7 times to sperm suspension to and 40 .mu.l of isotonic TALP achieve a final glycerol medium to 100 .mu.l of sperm sus- concentration, 0.125 M. pension with 1 M glycerol; (2) After centrifugation, 710 centrifuge the cell suspension .mu.l of supernatant was taken at 400 g for 5-7 minutes.; (3) off. Remaining cell sus- take off 170 .mu.l of the super- pension volume is 90 .mu.l natant; Stepwise add 10, 20 and 60 .mu.l of isotonic solution to the remaining 30 ul of sperm suspension. After the above 7 steps dilution, the glycerol concentration in the sperm suspension is 0.125 M. The final suspension volume is 90 .mu.l _____

CLPR:
4. The method of claim 1, wherein said cryoprotectant comprises glycerol.

CLPR:
12. The method of claim 11, wherein said cryoprotectant is selected from the group consisting of dimethyl sulfoxide, glycerol, and ethylene glycol.

CCOR:
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ORPL:
Cryobiology, vol. 26, issued 1989, Fiser et al., "The Effect of Glycerol-Related Osmotic Changes on Post-Thaw Motility and Acrosomal Integrity of Ram Spermotozoa", pp. 64-69.

ORPL:
Cryobiology, vol. 29, issued 1992, Gao et al., "Glycerol Permeability of Human Spermatozoa and its Activation Energy", pp. 657-667.

ORPL:
Biology of Reproduction, vol. 49, issued 1993, Gao et al., "Hyperosmotic Tolerance of Human Spermatozoa: Separate Effects on Glycerol, Sodium Chloride, and Sucrose on Spermolysis", pp. 112-113.

File: USPT Dec 23, 1997

L6: Entry 10 of 20

DOCUMENT-IDENTIFIER: US 5700632 A
TITLE: General method to quickly remove cryoprotectants from animal cells while maintaining viability

BSPU:
(1) the method is usable for other cryoprotectants besides the commonly used glycerol, dimethyl sulfoxide, or ethylene glycol,

DEPR:
The first two equations respectively describe total membrane volume flux (J.sub.v) and transmembrane permeable solute flux (J.sub.CPA). Assuming the solution consists of a single permeable solute (here, CPA) and other impermeable solutes (i.e. salt), the mathematical equations are: ##EQU1## where J.sub.v =total volume flux, V=cell volume, t=time, N=mole number of the solute, A.sub.c =cell surface area, L.sub.p =water permeability coefficient of the cell membrane, C=concentration of solute, J.sub.CPA =CPA flux across the cell membrane, superscript e=extracellular, superscript i=intracellular, C.sub.CPA =average CPA concentration of extracellular and intracellular concentrations. Furthermore, R=gas constant, T=absolute temperature, P.sub.CPA =CPA permeability coefficient of the cell membrane, and .sigma.=the reflection coefficient of the particular CPA. The reflection coefficient is generally specific to a particular cryoprotectant and represents the opposing actions of water and cryoprotectant moving in opposite directions through the cell membrane. Typically it is assumed to be 1. For example, using glycerol as the CPA, a value of 0.7 to 1 has only insignificant effect on the predictions found using this model. However, it must be remembered that its exact value is dependent on the cryoprotectant utilized and is a point where the user may fine tune the model for their particular use. Using the devices and methods discussed further in this patent, a more exact value can be determined.

DEPR:
Examples of the physical values for human sperm needed are shown in Table 1. Similar values for other species of sperm are within the skill in the art to obtain. For example, the permeability coefficient of the cryoprotective agent can be determined using procedures as disclosed in Glycerol Permeability of Human Spermatozoa and its Activation Energy, by Gao, Mazur, Kleinhans, Watson, Noiles, and Critser, Cryobiology 29, 657-667, the disclosure of which is hereby incorporated by reference into this specification; or, the permeability coefficient of water can be determined using procedures as disclosed in Determination of Water Permeability Coefficient for Human Spermatozoa and its Activation Energy, by Noiles, Mazur, Watson, Kleinhans, and Critser; Biology of Reproduction 48, 99-109 (1993) the disclosure of which is also hereby incorporated by reference into this specification.

DEPR:
Human RBC Preparation: Human blood was obtained by venipuncture from three healthy donors into 10 cc vacutainer tubes with acid citrate dextrose (SCD) anticoagulant. Cells were washed three times by centrifugation at 1000.times.g for five minutes each in PBS (pH=7.4) prior to use.

DEPR:
EPR sample preparation: Two .mu.l of 50 mM tempone, 10 m .mu.l of 250 mM CrOx, 28 .mu.l of sodium chloride solution (83, 153, 345, 694, 1217, or 2270 mOsm),

and 10 .mu.l packed sperm cells in TALP (centrifuged at 400.times.g for 10 minutes) were combined in a 5 ml sterile culture tube. The combined solution had a total volume of 50 .mu.l containing final concentrations of 2 mM tempone, 50 mM CrOx, and a total osmotic concentration of 250, 290, 400, 600, 900, or 1500 mOsm. The contents were mixed manually by tapping the tube. Ten .mu.l of each sample was transferred to another tube for trypan blue staining (10 .mu.l sample and 10 .mu.l 2@ trypan blue in isotonic saline, incubated for 10 min.) and cell counting of membrane intact cells on a standard hemocytometer under a light microscope which yields the sample cell concentration C.sub.c. The remaining sample was drawn by capillary action into a 50 .mu.l disposable micropipet (Clay Adams #4622, Parsnippany, N.J.) and sealed with Crioseal (Monoject Scientific, St. Louis, Mo.) for the EPR measurement. All the final osmotic concentrations were determined by linear addition of the individual osmolalities of the sodium chloride and CrOx (non-permeating solute). The osmolality of tempone was not included in the final concentration because it is a cell membrane permeable solute and has a very low concentration. The final strength of each solution was checked on a freezing point depression osmometer (Advanced DigiMatic Osmometer, Model 3D2) yielding agreement within 3%. The high concentration of tempone, 2 mM, was chosen to optimize signal strength and leads to some spin-spin line broadening.

DEPR:
The human semen used in this example was obtained by masterbation from healthy donors after at least two days of sexual abstinence. The samples were allowed to liquefy in an incubator for 1 hour, at 37.degree. C., in high humidity, and in 5% CO.sub.2 and 95% air. A swim-up procedure was performed to separate motile sperm from immotile sperm. The motile sperm suspensions were centrifuged at 400.times.g for 7 minutes and then were resuspended in isotonic TL-Hepes medium, that is, HEPES-buffered TALP medium (286-290 mOsm) supplemented with Pyruvate (0.01 mg/ml) and BSA (4 mg/ml), at a cell concentration of 1.times.10.sup.9 sperm/ml.

DEPR:
A similar pattern, with the sperm's volume first shrinking or swelling and then recovering, takes place during the CPA addition or removal process from the sperm. Generally, when a cell is placed in a solution that is hyperosmotic with respect to the permeating solute (e.g. glycerol) but isotonic with respect to the impermeable salts, it first shrinks because of the osmotic efflux of intracellular water and then increases in volume as the solute (e.g. glycerol) permeates and as water concomitantly reenters the cell. When cells with CPA are exposed to an isotonic solution, they will swell because of osmotic influx of extracellular water and then decrease in volume as the CPA diffuses out of the sperm and as water concomitantly moves out.

DEPR:
The sperm's volume changes during a 1-step addition and a 1-step removal of 9.5-2M glycerol were calculated from computer simulation and are respectively shown in FIGS. 7 and 8. The higher the glycerol concentration, the longer the time period taken for the sperm volume recovery. However, typically it takes but a few seconds for human sperm to achieve their osmotic equilibration volumes. This means that the sperm experience the shrunken or swollen states for only an order of seconds during the addition or removal of glycerol. Therefore, the information concerning post-anisosmotic tolerance of the sperm returned to isotonic conditions after a short time exposure (an order of seconds) to the anisosmotic conditions is particularly important for designing optimal CPA addition/removal procedures to prevent sperm osmotic injury.

DEPR:
The kinetics of water and glycerol transport across the sperm membrane were modeled using standard computing techniques that are well within the skill of the art of this invention and using Equations 1 to 4. Two preferred schemes of CPA addition, Fixed-Volume-Step (FVS) and Fixed-Molarity-Step (FMS), were analyzed in this regard and are presented below.

DEPR:
Referring to FIG. 10, there is shown the calculated sperm volume change arising during a one-step, a two-step, and a four-step addition of glycerol to achieve a final 1M glycerol concentration at 22.degree. C. using an FMS addition.

DEPR:
Referring to FIG. 11, there is shown a comparison between a four-step FVS addition of glycerol and a four-step FMS of glycerol.

DEPR:
From FIGS. 9 to 11, a one-step addition of glycerol to sperm is predicted to cause a 10% to 20% sperm motility loss. This loss is predicted to occur because the minimum volume which the sperm can attain during this addition is approximately 72% of the original cell volume, a value less than the minimally acceptable LVL of 75% previously determined above. In contrast, a four-step FVS or FMS addition of glycerol was predicted to greatly reduce such sperm motility loss (less than 5%).

DEPR:
Referring to FIG. 12, there is shown a one-step removal of glycerol. This removal was predicted to cause as high as 70% motility loss because the maximum cell volume during the glycerol removal was calculated to be over 1.6 times larger than the isotonic cell volume, much higher than the upper volume limit of the sperm.

DEPR:
Referring to FIG. 14, there is shown a comparison between the eight-step FMS and the eight-step FVS removal procedures. The eight-step FMS removal was predicted to prevent sperm motility loss over the FVS procedure. An eight-step FVS removal was predicted to cause a maximum cell swelling of over 1.2 times higher than isotonic cell volume while FMS removal was predicted to be much lower than the UVL, indicating that the eight-step FVS removal is not as good as eight-step FMS removal. Also apparent from the computer simulation was the prediction that the human sperm would rapidly achieve an osmotic equilibrium (within seconds) during the 1-step or stepwise glycerol addition or removal. This further indicated that only a short time interval between steps of glycerol addition or removal was required.

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In sum, a four-step FMS addition and an eight-step FMS removal of glycerol were predicted to be optimal protocols to prevent sperm motility loss in human sperm using the foregoing equations.

DEPR:
A two-step removal of CPA from the cells using a nonpermeating solute as an osmotic buffer has been previously used to avoid the osmotic injury to other cell types. The detailed procedure is: (1) the CPA is directly removed from the cells by transferring cells to a hyperosmotic medium (osmotic buffer) containing no CPA but only nonpermeating solutes, and then (2) the cells are directly transferred to an isotonic solution. It has been known that 600 mOsm is the hyperosmotic upper tolerance limit for human sperm (FIG. 9). Therefore, osmolality of the osmotic buffer medium should not be over 600 mOsin. Under this limit, a hyperosmolality of 600 mOsm is expected to provide the maximum "buffer effect" to reduce the sperm volume swelling during the first step of the "two-step" procedure for glycerol removal. Sperm volume change during the 2-step glycerol (1M) removal process using a 600 mOsm buffer medium was calculated and shown in FIG. 15. It was predicted that maximum volume of the sperm would achieve 1.25 times of the isotonic cell volume, which is higher than the UVL of the sperm, and might cause over 50% sperm motility loss.

DEPR:
TL-HEPES medium with 2M glycerol was either one-step or stepwise added to an equal volume of the isotonic sperm suspension to achieve a final 1M glycerol concentration at 22.degree. C. Glycerol in the sperm was removed by dilution

either by a one-step or stepwise addition of the TL-HEPES medium with or without an osmotic buffer (sucrose) placed into the cell suspension. The detailed procedures for the glycerol addition and removal are described in Tables 2-5. Sperm motility before, during, and after the different glycerol addition and removal procedures was measured by CASA. The membrane integrity of the sperm was determined by the dual staining technique and flow cytometry.

DEPR:
Add 2000 .mu.l of isotonic solution directly to 100 .mu.l of cell suspension with 1M glycerol.

DEPR:
Glycerol was experimelltally added to or removed from the human sperm using the stepwise procedures as predicted from the computer simulation. Percent motility of the human sperm after one step or a four-step FMS addition of glycerol (Table 2) is shown in FIG. 16. One step addition created approximately 20% sperm motility loss while the four-step addition, less than 8%. FIG. 17 shows effects of different glycerol removal procedures (Table 3) on the human sperm motility loss. Less than 30% motile sperm kept motility after one-step removal of glycerol while majority of sperm (over 92%) maintained the motility after the eight-step FMS removal. The motility loss caused by a two-step removal of glycerol using sucrose as a non-permeating buffer (total osmolality of the buffer medium was 600 mOsm) was close to 45%. Thirty-five percent of the sperm lost motility after a eight-step FVS removal of glycerol. The experimental results agree well with the predictions generated from computer simulation.

DEPR:
FIG. 18 shows the membrane integrity of the human sperm in 1M glycerol solution or after addition and removal of 1M glycerol by the different procedures. The sperm appeared to maintain membrane integrity under all experimental conditions.

DEPR:
This example presented two CPA addition or removal schemes (FVS and FMS). As the example shows, the Fixed-Molarity-Step is preferable to reduce osmotic injury over the Fixed-Volume-Step. In particular, for human sperm, the example shows that a four-step FMS addition of glycerol to the sperm and an eight-step FMS removal of glycerol from the sperm were predicted to be optimal, which was confirmed upon implementation. Upon reviewing each scheme, the minimum/maximum cell volumes after each step of FVS addition/removal was uneven or unequal, some of which exceeded the LVL and UVL of the sperm. In contrast, minimum/maximum cell volumes after each step of FMS addition/removal of glycerol were shown to be relative even (FIGS. 11 and 14). For a fixed number of steps, the extent of cell volume change during CPA addition/removal using the FMS scheme is much smaller than that using the FVS scheme (also see FIGS. 11 and 14).

DEPR:
A corresponding computer program using a finite difference method to calculate the above equations is used to simulate the mass transfer process in the system. The values of f, L2, C.sub.f, C.sub.i, L1, D1, D2 and h.sub.D can be used as control factors or adjustable parameters in the computer simulation. Using this computer program coupled with the computer program to calculate cell membrane transport (described by cell membrane transport equations 1 to 4, presented earlier), one may calculate the kinetic decrease of CPA concentration in the cell suspension and in cells as well as corresponding cell volume expansion. The optimal parameters to be used to design the system are simply those combinations which allow the quickest removal of the CPA but do not cause cell volume excursion beyond cell tolerance limits. One set of acceptable values of parameters for designing the diffusion washing device for human sperm was predicted from computer simulations: pore size: 0.65 .mu.m in diameter, 70% pore area, 0.5 mm of the chamber space, i.e. L value. Calculated kinetic changes of glycerol concentration (initial 1M) at the chamber position

A (center, see FIG. 19) and B (internal surface of porous membrane, see FIG. 19) are shown in FIG. 21, and the corresponding cell volume changes are calculated and predicted to be below the cell tolerence limit (1.1 times isotonic sperm volume), as shown in FIG. 22. Using a diffusion device design based on the computer simulation results above, over 95% of 1M glycerol was removed from the human sperm cells within 10 minutes without significant cell clumping, loss in motility or membrane integrity, or curling of sperm tails; this result was consistent with theoretical predictions.

DEPL:
where M.sub.s =CPA concentration in cell suspension (molarity) after n-1 step dilutions, M.sub.o =CPA concentration in initial cell suspension (molarity), n=total number of steps, i=the ith step addition, V.sub.o =original volume of cell suspension (ml) and V.sub.i =volume of isotonic solution added into the cell suspension at the ith step. After n-1 steps of adding isotonic solution into the cell suspension, the diluted cell suspension is centrifuged, for example, at 400 g for 5-7 minutes., and then the cell pellet is resuspended in isotonic solution to make the last (nth) step dilution.

DEPC:
Prediction of Optimal Conditions for Glycerol Addition or Removal

DEPV:
Procedures used in 2-step removal of 1M glycerol from the human sperm using sucrose as an osmotic buffer

DEPV:
(1) Add 2000 .mu.l of sucrose buffer medium (TALP+sucrose, 600 mOsm) to 100 .mu.l of sperm suspension with 1M glycerol; (2) centrifuge the suspension (400 g for 7 minutes) and take off the supernatant; and (3) resuspend cell pellet with 500 .mu.l isotonic TALP medium.

DETL:
TABLE 1 _____ Known characeristics of human spermatozoa _____ Surface area (A) 120 um.sup.2 Volume (V) 34 um.sup.3 Osmotically inactive volume (V.sub.b) 16.6 um.sup.3 Water permeability coefficient (Lp) 2.16 um/minutes/atm (22.degree. C.) Glycerol permeability coefficient(P.sub.CPA) 1.1 .times. 10.sup.-3 cm/minutes (22.degree. C.) _____

DETL:
TABLE 2 _____ Procedures used in 4-step addition of 1 ml of 2M glycerol solution of 1 ml of isotonic sperm suspension FVS FMS _____ Add 0.25 ml of 2M glycerol Stepwise add 0.14, 0.19, 0.27 4 times to 1 ml isotonic and 0.4 ml of 2M glycerol sperm suspension to isotonic sperm suspension

_____

DETL:
TABLE 3 _____ Procedures used in 1-step and 8-step removal of 1M glycerol from the human sperm 8-Step Dilution FVS FMS _____ Add 100 .mu.l of isotonic TALP Stepwise add 14.3, 19, 26.6, 7 times to sperm suspension to and 40 .mu.l of isotonic TALP achieve a final glycerol medium to 100 .mu.l of sperm concentration, 0.125M. suspension with 1M glycerol; After centrifugation, 710 (2) centrifuge the cell .mu.l of supernatant was taken suspension at 400 g for off. Remaining cell sus- 5-7 minutes.; (3) take off pension volume is 90 .mu.l 170 .mu.l of the supernatant; Stepwise add 10, 20 and 60 .mu.l of isotonic solution to the remaining 30 ul of sperm suspension. After the above 7 steps dilution, the glycerol concentration in the sperm suspension is 0.125M. The final suspension volume is 90 .mu.l _____

CLPR:
4. The method of claim 1, wherein said cryoprotectant comprises glycerol.

CLPR:
14. The method of claim 11, wherein said cryoprotectant is selected from the group consisting of dimethyl sulfoxide, glycerol, and ethylene glycol.

CLPR:
23. The method of claim 19, wherein said cryoprotectant is selected from the group consisting of dimethyl sulfoxide, glycerol, and ethylene glycol.

CLPR:
28. The method of claim 24, wherein said cryoprotectant is selected from the group consisting of dimethyl sulfoxide, glycerol, and ethylene glycol.

CCOR:
435/2

ORPL:
Cryobiology, vol. 26, issued 1989, Fiser et al., "The Effect of Glycerol-Related Osmotic Changes on Post-Thaw Motility and Acrosomal Integrity of Ram Spermatozoa", pp. 64-69.

ORPL:
Cryobiology, vol. 29, issued 1992, Gao et al., "Glycerol Permeability of Human Spermatozoa and its Activation Energy", pp. 657-667.

ORPL:
Biology of Reproduction, vol. 49, issued 1993, Gao et al., "Hyperosmotic Tolerance of Human Spermatozoa: Separate Effects on Glycerol, Sodium Chloride, and Sucrose on Spermolysis", pp. 112-123.

WEST

End of Result Set

Generate Collection

L12: Entry 1 of 1 File: USPT Oct 31, 2000

DOCUMENT-IDENTIFIER: US 6140121 A
TITLE: Methods and compositions to improve germ cell and embryo survival and function

DEPR:
Extending sperm is used to resuspend a sperm pellet following isolation or washing, to dilute a semen sample, to dilute a culture of sperm, and the like. In this way, sperm are placed into a medium suitable for a variety of procedures, including culture, insemination, assays of fertilization potential as described herein, in vitro fertilization, freezing, intrauterine insemination, cervical cap insemination, and the like. The sperm may be added to the medium or the medium may be added to the sperm. Preferably, the medium contains gum guar, gum arabic, pectin or galacturonic acid, although another PCAGH may be used. In other aspects of this invention, methods are provided for the culture of such extended sperm to increase their survival during holding or culture at a range of temperatures from about room temperature (e.g., 20.degree. C.) to about body temperature (e.g., 37.degree. C. or 39.degree. C.). This includes culture of sperm in toxicity screen tests and the holding of sperm for sorting into X and Y chromosome-containing fractions by flow cytometry for generating sexed offspring. In other aspects of this invention, sperm extending medium is used for preparing sperm for direct insemination, cryopreservation, and for intracytoplasmic sperm injection (ICSI) which requires a more viscous media to slow motile sperm down for pick-up by the transfer pipette for injection into the egg. In ICSI, the medium contains PCAGH at higher levels than a routine extender medium (ie., 1% arabic acid or 5% gum arabic) to increase viscosity. A viscous solution of PCAGH also has a positive effect on sperm function by limiting membrane damage and possible chromatin breakdown during in vitro handling. Additional embodiments include encapsulation of the sperm (Munkittrick et al., J. Diary Sci. 75:725-731) in an alginate or protamine sulfate microcapsule containing PCAGH, such as pectin at 0.05%. Encapsulation allows for shedding of sperm over an increased time frame so that insemination does not have to be as well timed with ovulation. PCAGH stabilizes sperm membranes from breakdown observed with current procedures.

WEST

Generate Collection

L13: Entry 1 of 20 File: USPT Oct 31, 2000

DOCUMENT-IDENTIFIER: US 6140121 A
TITLE: Methods and compositions to improve germ cell and embryo survival and function

BSPR:
One common procedure used in sperm collection is washing sperm cells. Washing sperm prior to its use in assisted reproduction technologies is important for a variety of reasons. An ejaculate contains seminal plasma in addition to sperm cells, and the sugars and proteins in seminal plasma can be toxic to sperm cells after ejaculation. Also, sperm samples that have been frozen contain cryopreservation media which needs to be washed from the sperm cells prior to insemination in the female of some species, particularly birds and women. For all species, cryopreservative media cause lipid membrane peroxidation (LPO) and degeneration of the sperm after thawing. Generally, washing involves centrifuging a sample of semen or thawed sperm through a diluting wash media, which allows collection of a sperm-rich pellet. Although a very common procedure, centrifugation itself can cause sperm lipid peroxidation and membrane breakdown.

BSPR:
Once sperm have been washed or isolated, they are then extended (or diluted) in culture or holding media for a variety of uses. Existing sperm culture techniques result in losses of motile sperm and also damage sperm DNA over time in culture. Although sperm survive for days in the females of most species, sperm survival in culture is typically only half as long as that seen in vivo, and sperm from males with poor quality ejaculates may survive for even shorter time periods in culture. Much of this damage is due to lipid peroxidation of the membrane and DNA or chromatin breakdown. Sperm are extended in media for use in sperm analysis and diagnostic tests; assisted reproduction technologies, such as IVF, gamete intrafallopian transfer, or ICSI; insemination into the female; and holding prior to cryopreservation. Each of these uses for extended or diluted sperm requires a somewhat different formulation of basal medium; however, in all cases sperm survival is suboptimal outside of the female reproductive tract.

DRPR:
FIGS. 11A-11D are flow cytometry profiles of DNA from sperm frozen with PCAGH (A) and (B) or egg yolk buffer (C) and (D) extenders.

DEPR:
Extending sperm is used to resuspend a sperm pellet following isolation or washing, to dilute a semen sample, to dilute a culture of sperm, and the like. In this way, sperm are placed into a medium suitable for a variety of procedures, including culture, insemination, assays of fertilization potential as described herein, in vitro fertilization, freezing, intrauterine insemination, cervical cap insemination, and the like. The sperm may be added to the medium or the medium may be added to the sperm. Preferably, the medium contains gum guar, gum arabic, pectin or galacturonic acid, although another PCAGH may be used. In other aspects of this invention, methods are provided for the culture of such extended sperm to increase their survival during holding or culture at a range of temperatures from about room temperature (e.g., 20.degree. C.) to about body temperature (e.g., 37.degree. C. or 39.degree. C.). This includes culture of sperm in toxicity screen tests and

the holding of sperm for sorting into X and Y chromosome-containing fractions by flow cytometry for generating sexed offspring. In other aspects of this invention, sperm extending medium is used for preparing sperm for direct insemination, cryopreservation, and for intracytoplasmic sperm injection (ICSI) which requires a more viscous media to slow motile sperm down for pick-up by the transfer pipette for injection into the egg. In ICSI, the medium contains PCAGH at higher levels than a routine extender medium (ie., 1% arabic acid or 5% gum arabic) to increase viscosity. A viscous solution of PCAGH also has a positive effect on sperm function by limiting membrane damage and possible chromatin breakdown during in vitro handling. Additional embodiments include encapsulation of the sperm (Munkittrick et al., J. Diary Sci. 75:725-731) in an alginate or protamine sulfate microcapsule containing PCAGH, such as pectin at 0.05%. Encapsulation allows for shedding of sperm over an increased time frame so that insemination does not have to be as well timed with ovulation. PCAGH stabilizes sperm membranes from breakdown observed with current procedures.

DEPR:
The compositions and methods of the present invention increase fertility of animals. These methods are generally applicable to many species, including human, bovine, canine, equine, porcine, ovine, avian, rodent and others. Although useful whenever fertilization is desired, the present invention has particular use in animals and humans that have a fertilization dysfunction in order to increase the likelihood of conception. Such dysfunctions include low sperm count, reduced motility of sperm, and abnormal morphology of sperm. In addition to these dysfunctions, the methods and compositions of the present invention are useful in artificial insemination procedures. Often, in commercial breedings, the male and female are geographically distant requiring the shipment of sperm for insemination. Because of the extended period of time between ejaculation and insemination, shipment in refrigerated or frozen state is necessary. As well, for particularly valuable or rare animals, long-term storage may be desirable. For humans, geographical distance or time considerations may necessitate storage of sperm. Men with diseases where radiation treatment is part of therapy or prior to vasectomies may desire to have sperm stored for future use. After frozen storage, cells are often cultured during end use. Survival and health of the cells in culture have been shown to be improved by addition of a PCAGH to the cryopreservative medium.

ORPL:
G. Maisse, "Comparison of different carbohydrates for the cryopreservation of rainbow trout (Oncorhynchus mykiss) sperm," CAB International, Abstract No. 940104586, 1994 & see also Aquatic Living Resources 7 (3):217-219, 1994.

WEST

Generate Collection

L13: Entry 7 of 20 File: USPT Jun 8, 1999

DOCUMENT-IDENTIFIER: US 5910568 A
TITLE: Molecule involved in binding of sperm to egg surfaces and procedures for use of this molecule to enhance or decrease potential fertility

ABPL:
A purified polypeptide which provides for initial binding of sperm to oocyte investments and has an active amino acid sequence of SEQ ID NO:12 (Cys-Gln-Ser-Leu-Gln-Glu-Tyr-Leu-Ala-Glu-Gln-Asn-Gln-Arg-Gln-Leu-Glu-Ser-A sn-Lys-Ile-Pro-Glu-Val-Asp-Leu-Ala-Arg-Val-Val-Ala-Pro-Phe-Met-Ser-Asn-Ile-P ro-Leu-Leu-Leu-Tyr-Pro-Gln-Asp-Arg-Pro -Arg-Ser-Gln-Pro-Gln-Pro-Lys-Ala-Asn-Glu-Asp-Val-Cys); or SEQ ID NO:13 (Cys-Glu-Ser-Leu-Gln-Lys-His-Leu-Ala-Glu-Leu-Asn-His-Gln-Lys-Gln-Leu-Glu-S er-Asn-Lys-Ile-Pro-Glu-Leu -Asp-Met-Thr-Glu-Val-Val-Ala-Pro-Phe-Met-Ala-Asn-Ile-Pro-Leu-Leu-Leu-Tyr-P ro-Gln-Asp-Gly-Pro-Arg-Ser-Lys-Pro-Gln -Pro-Lys-Asp-Asn-Gly-Asp-Val-Cys); or the shorter but biologically active SEQ ID NO:1 and SEQ ID NO:9 (Tyr-Pro-Gln-Asp-Arg-X-Arg-Ser-Gln-Pro-Gln-Pro-Lys-Ala-Asn, where X is Thr or Pro). The polypeptide is useful in improving sperm-egg binding by fresh sperm, restoring sperm-egg binding following a cryopreservation cycle, enhancing fertilizing potential, and producing antibodies to determine fertilizing potential of sperm, determining sperm-binding sites on egg investments, and contraception.

BSPR:
The present invention is a purified polypeptide (termed universal primary sperm-egg binding protein, or UPSEBP) which provides for initial bonding of sperm to oocyte investments and uses of the polypeptide. The inventive polypeptide has biological activity in a variety of avian and mammalian species and has active sites within the amino acid sequence embodied in SEQ ID NO:12 which is Cys-Gln-Ser-Leu-Gln-Glu-Tyr-Leu-Ala-Glu-Gln-Asn-Gln-Arg-Gln-Leu-Glu-Ser-As n-Lys-Ile-Pro-Glu-Val-Asp-Leu-Ala-Arg -Val-Val-Ala-Pro-Phe-Met-Ser-Asn-Ile-Pro-Leu-Leu-Leu-Tyr-Pro-Gln-Asp-Arg-P ro-Arg-Ser-Gln-Pro-Gln-Pro-Lys-Ala-Asn-Glu-Asp-Val-Cys. One of these active sites is within the portion of the amino acid sequence embodied in SEQ ID NO:9 or Tyr-Pro-Gln-Asp-Arg-X-Arg-Ser-Gln-Pro-Gln-Pro-Lys-Ala-Asn, where X is Thr or Pro. The tertiary structure of SEQ ID NO:12 or a similar sequence affects biological activity. Uses of the synthetic or natural polypeptide include in vitro treatment of sperm to restore fertilizing capacity for some samples of thawed cryopreserved sperm or to enhance fertilizing potential of some fresh sperm, intra-vaginal treatment of sperm to enhance sperm-egg binding and increase probability of fertilization, and use in an in vitro assay to determine fertilizing potential of sperm. Antibodies to the polypeptide are also useful for in vitro assays to determine potential fertility of sperm or number of sperm-binding sites on an egg investment or in vivo as a contraceptive.

DRPR:
FIG. 1 compares the number of sperm bound before and after cryopreservation, treated with UPSEBP and untreated to fresh sperm;

DRPR:
FIG. 7a shows the restorative capability of rooster UPSEBP (native protein

extracted from rooster sperm) and rat SGP-1 on binding of thawed cryopreserved turkey sperm;

DRPR:
FIG. 7b shows the restorative capability of turkey UPSEBP (native protein extracted from turkey sperm) on binding of thawed cryopreserved rooster sperm and thawed cryopreserved turkey sperm;

DRPR:
FIG. 9 shows the restorative capability of rooster UPSEBP (native protein extracted from rooster sperm) on binding of thawed cryopreserved stallion sperm.

DEPR:
Freshly collected rooster sperm were cryopreserved, thawed and processed to remove glycerol. One portion of the thawed sperm received crude UPSEBP at a concentration of 2 .mu.g protein per 10.sup.6 sperm. Samples containing 1-10.times.10.sup.6 sperm were placed into wells coated with an extract of hen's egg membrane and incubated 180 minutes. Non-bound sperm were removed by washing, and bound sperm were stained with diamido-2-phenylindole and counted using an epifluorescent microscope. The results presented in FIG. 1 demonstrate the ability of UPSEBP to restore the capability of frozen-thawed rooster sperm to bind to hen's egg membrane protein in comparison to unprocessed sperm.

DEPR:
Sperm in a sample of pooled semen were used to obtain fertility data before cryopreservation (i.e., fresh) and after cryopreservation and post-thaw processing to slowly remove glycerol (frozen-thawed). One portion of the thawed sperm was suspended in Minnesota-A buffer alone, and the second was treated with Minnesota-A buffer containing crude UPSEBP at 2 .mu.g protein per 10.sup.6 sperm. As shown in FIG. 4, fertility of the sperm processed in UPSEBP was double that for sperm not treated with UPSEBP and approached the level of fresh sperm. Hatchability of eggs artificially fertilized by sperm treated with UPSEBP increased from 18 to 25 percent.

DEPR:
Rooster sperm subjected to a cryopreservation cycle were treated with increasing amounts of partially purified UPSEBP and subject to a standard binding assay, together with aliquots of unfrozen (fresh) sperm. FIG. 6 shows that treatment of frozen-thawed rooster sperm with 0.04-4.0 .mu.g UPSEBP per 10.sup.6 sperm partially restored the sperm's capability to bind to the membrane of a hen's egg.

DEPR:
A primary antibody was produced in two rabbits against SEQ ID NO:5, linked via the cysteine amino acid to keyhole limpet, with use of standard adjuvants and techniques. The immune serum was harvested and used as a primary antibody to label rooster sperm, fixed in low ionic strength buffer using paraformaldehyde and processed by standard techniques, after which fluorescein isothiocyanate-labeled goat-antisheep gama globulin was used as a second antibody. Analyses by flow cytometry revealed the percent of sperm as labeled, and visual examinations by epifluorescence microscopy revealed localization of the antibodies to the head region. A similar antibody was prepared against SEQ ID NO:12.

WEST

Generate Collection

L13: Entry 2 of 20 File: USPT Aug 15, 2000

DOCUMENT-IDENTIFIER: US 6103483 A
TITLE: Molecule involved in binding of sperm to egg surfaces and procedures for use of this molecule to enhance or decrease potential fertility

ABPL:
A purified polypeptide which provides for initial binding of sperm to oocyte investments and has an active amino acid sequence of SEQ ID NO:12 (Cys-Gln-Ser-Leu-Gln-Glu-Tyr-Leu-Ala-Glu-Gln-Asn-Gln-Arg-Gln-Leu-Glu-Ser-Asn-Lys-Ile-Pro-Glu-Val-Asp-Leu-Ala-Arg-Val-Val-Ala-Pro-Phe-Met-Ser-Asn-Ile-Pro-Leu-Leu-Leu-Tyr-Pro-Gln-Asp-Arg-Pro-Arg-Ser-Gln-Pro-Gln-Pro-Lys-Ala-Asn-Glu-Asp-Val-Cys); or SEQ ID NO:13 (Cys-Glu-Ser-Leu-Gln-Lys-His-Leu-Ala-Glu-Leu-Asn-His-Gln-Lys-Gln-Leu-Glu-Ser-Asn-Lys-Ile-Pro-Glu-Leu-Asp-Met-Thr-Glu-Val-Val-Ala-Pro-Phe-Met-Ala-Asn-Ile-Pro-Leu-Leu-Leu-Tyr-Pro-Gln-Asp-Gly-Pro-Arg-Ser-Lys-Pro-Gln-Pro-Lys-Asp-Asn-Gly-Asp-Val-Cys); or the shorter but biologically active SEQ ID NO:1 and SEQ ID NO:9 (Tyr-Pro-Gln-Asp-Arg-X-Arg-Ser-Gln-Pro-Gln-Pro-Lys-Ala-Asn, where X is Thr or Pro). The polypeptide is useful in improving sperm-egg binding by fresh sperm, restoring sperm-egg binding following a cryopreservation cycle, enhancing fertilizing potential, and producing antibodies to determine fertilizing potential of sperm, determining sperm-binding sites on egg investments, and contraception.

BSPR:
include in vitro treatment of sperm to restore fertilizing capacity for some samples of thawed cryopreserved sperm or to enhance fertilizing potential of some fresh sperm, intra-vaginal treatment of sperm to enhance sperm-egg binding and increase probability of fertilization, and use in an in vitro assay to determine fertilizing potential of sperm. Antibodies to the polypeptide are also useful for in vitro assays to determine potential fertility of sperm or number of sperm-binding sites on an egg investment or in vivo as a contraceptive.

DRPR:
FIG. 1 compares the number of sperm bound before and after cryopreservation, treated with UPSEBP and untreated to fresh sperm;

DRPR:
FIG. 7a shows the restorative capability of rooster UPSEBP (native protein extracted from rooster sperm) and rat SGP-1 on binding of thawed cryopreserved turkey sperm;

DRPR:
FIG. 7b shows the restorative capability of turkey UPSEBP (native protein extracted from turkey sperm) on binding of thawed cryopreserved rooster sperm and thawed cryopreserved turkey sperm;

DRPR:
FIG. 9 shows the restorative capability of rooster UPSEBP (native protein extracted from rooster sperm) on binding of thawed cryopreserved stallion sperm.

DEPR:
Freshly collected rooster sperm were cryopreserved, thawed and processed to

remove glycerol. One portion of the thawed sperm received crude UPSEBP at a concentration of 2 .mu.g protein per 10.sup.6 sperm. Samples containing 1-10.times.10.sup.6 sperm were placed into wells coated with an extract of hen's egg membrane and incubated 180 minutes. Non-bound sperm were removed by washing, and bound sperm were stained with diamido-2-phenylindole and counted using an epifluorescent microscope. The results presented in FIG. 1 demonstrate the ability of UPSEBP to restore the capability of frozen-thawed rooster sperm to bind to hen's egg membrane protein in comparison to unprocessed sperm.

DEPR:
Sperm in a sample of pooled semen were used to obtain fertility data before cryopreservation (i.e., fresh) and after cryopreservation and post-thaw processing to slowly remove glycerol (frozen-thawed). One portion of the thawed sperm was suspended in Minnesota-A buffer alone, and the second was treated with Minnesota-A buffer containing crude UPSEBP at 2 .mu.g protein per 10.sup.6 sperm. As shown in FIG. 4, fertility of the sperm processed in UPSEBP was double that for sperm not treated with UPSEBP and approached the level of fresh sperm. Hatchability of eggs artificially fertilized by sperm treated with UPSEBP increased from 18 to 25 percent.

DEPR:
Rooster sperm subjected to a cryopreservation cycle were treated with increasing amounts of partially purified UPSEBP and subject to a standard binding assay, together with aliquots of unfrozen (fresh) sperm. FIG. 6 shows that treatment of frozen-thawed rooster sperm with 0.04-4.0 .mu.g UPSEBP per 10.sup.6 sperm partially restored the sperm's capability to bind to the membrane of a hen's egg.

DEPR:
A primary antibody was produced in two rabbits against SEQ ID NO:5, linked via the cysteine amino acid to keyhole limpet, with use of standard adjuvants and techniques. The immune serum was harvested and used as a primary antibody to label rooster sperm, fixed in low ionic strength buffer using paraformaldehyde and processed by standard techniques, after which fluorescein isothiocyanate-labeled goat-antisheep gama globulin was used as a second antibody. Analyses by flow cytometry revealed the percent of sperm as labeled, and visual examinations by epifluorescence microscopy revealed localization of the antibodies to the head region. A similar antibody was prepared against SEQ ID NO:12.

WEST

Generate Collection

L13: Entry 17 of 20 File: USPT Jan 21, 1997

DOCUMENT-IDENTIFIER: US 5595866 A
TITLE: Step-wise method to remove cryoprotectant from sperm

DEPR:
TL-HEPES medium with 2M glycerol was either one-step or stepwise added to an equal volume of the isotonic sperm suspension to achieve a final 1M glycerol concentration at 22.degree. C. Glycerol in the sperm was removed by dilution either by a one-step or stepwise addition of the TL-HEPES medium with or without an osmotic buffer (sucrose) placed into the cell suspension. The detailed procedures for the glycerol addition and removal are described in Tables 2-5. Sperm motility before, during, and after the different glycerol addition and removal procedures was measured by CASA. The membrane integrity of the sperm was determined by the dual staining technique and flow cytometry.

DEPR:
The addition of CPA to the sperm before cooling and its removal from sperm after warming are two of very important procedures in sperm cryopreservation. The present invention offers a new methodology to define optimal procedures to carry out either or both of these procedures in a fashion to reduce osmotic injury of the sperm. The calculated procedures were implemented and the results agree remarkably well with prediction.

DEPR:
Finally, it should be remembered that the effect of potential chemical toxicity of CPA on sperm cell viability is another reason causing sperm cell injury during the addition and removal of CPA for sperm cell cryopreservation. Given a CPA type and CPA concentration, it is generally accepted that the potential toxicity of the CPA to the cells is decreased with a decrease of cell exposure time to the CPA and a decrease in temperature.

WEST

Generate Collection

L13: Entry 13 of 20 File: USPT Nov 25, 1997

DOCUMENT-IDENTIFIER: US 5691133 A
TITLE: Method to quickly add cryoprotectants to sperm cells while maintaining viability

DEPR:
TL-HEPES medium with 2M glycerol was either one-step or stepwise added to an equal volume of the isotonic sperm suspension to achieve a final 1M glycerol concentration at 22.degree. C. Glycerol in the sperm was removed by dilution either by a one-step or stepwise addition of the TL HEPES medium with or without an osmotic buffer (sucrose) placed into the cell suspension. The detailed procedures for the glycerol addition and removal are described in Tables 2-5. Sperm motility before, during, and after the different glycerol addition and removal procedures was measured by CASA. The membrane integrity of the sperm was determined by the dual staining technique and flow cytometry.

DEPR:
The addition of CPA to the sperm before cooling and its removal from sperm after warming are two of very important procedures in sperm cryopreservation. The present invention offers a new methodology to define optimal procedures to carry out either or both of these procedures in a fashion to reduce osmotic injury of the sperm. The calculated procedures were implemented and the results agree remarkably well with prediction.

DEPR:
Finally, it should be remembered that the effect of potential chemical toxicity of CPA on sperm cell viability is another reason causing sperm cell injury during the addition and removal of CPA for sperm cell cryopreservation. Given a CPA type and CPA concentration, it is generally accepted that the potential toxicity of the CPA to the cells is decreased with a decrease of cell exposure time to the CPA and a decrease in temperature.

ORPL:
Acta Vet. Scand., vol. 32, No. 4, issued 1991, C.O. Bwanga, "Cryopreservation of Boar Sperm", pp. 431-453.

WEST

Generate Collection

L13: Entry 3 of 20 File: USPT Apr 25, 2000

DOCUMENT-IDENTIFIER: US 6054287 A
TITLE: Cell-type-specific methods and devices for the low temperature preservation of the cells of an animal species

DEPR:
TL-HEPES medium with 2M glycerol was either one-step or stepwise added to an equal volume of the isotonic sperm suspension to achieve a final 1M glycerol concentration at 22.degree. C. Glycerol in the sperm was removed by dilution either by a one-step or stepwise addition of the TL-HEPES medium with or without an osmotic buffer (sucrose) placed into the cell suspension. The detailed procedures for the glycerol addition and removal are described in Tables 2-5. Sperm motility before, during, and after the different glycerol addition and removal procedures was measured by CASA. The membrane integrity of the sperm was determined by the dual staining technique and flow cytometry.

DEPR:
The addition of CPA to the sperm before cooling and its removal from sperm after warming are two of very important procedures in sperm cryopreservation. The present invention offers a new methodology to define optimal procedures to carry out either or both of these procedures in a fashion to reduce osmotic injury of the sperm. The calculated procedures were implemented and the results agree remarkably well with prediction.

DEPR:
Finally, it should be remembered that the effect of potential chemical toxicity of CPA on sperm cell viability is another reason causing sperm cell injury during the addition and removal of CPA for sperm cell cryopreservation. Given a CPA type and CPA concentration, it is generally accepted that the potential toxicity of the CPA to the cells is decreased with a decrease of cell exposure time to the CPA and a decrease in temperature.

WEST

Generate Collection

L13: Entry 11 of 20 File: USPT May 19, 1998

DOCUMENT-IDENTIFIER: US 5753427 A
TITLE: General method to quickly add cryoprotectants to animal cells while maintaining viability

DEPR:
TL-HEPES medium with 2M glycerol was either one-step or stepwise added to an equal volume of the isotonic sperm suspension to achieve a final 1M glycerol concentration at 22.degree. C. Glycerol in the sperm was removed by dilution either by a one-step or stepwise addition of the TL-HEPES medium with or without an osmotic buffer (sucrose) placed into the cell suspension. The detailed procedures for the glycerol addition and removal are described in Tables 2-5. Sperm motility before, during, and after the different glycerol addition and removal procedures was measured by CASA. The membrane integrity of the sperm was determined by the dual staining technique and flow cytometry.

DEPR:
The addition of CPA to the sperm before cooling and its removal from sperm after warming are two of very important procedures in sperm cryopreservation. The present invention offers a new methodology to define optimal procedures to carry out either or both of these procedures in a fashion to reduce osmotic injury of the sperm. The calculated procedures were implemented and the results agree remarkably well with prediction.

DEPR:
Finally, it should be remembered that the effect of potential chemical toxicity of CPA on sperm cell viability is another reason causing sperm cell injury during the addition and removal of CPA for sperm cell cryopreservation. Given a CPA type and CPA concentration, it is generally accepted that the potential toxicity of the CPA to the cells is decreased with a decrease of cell exposure time to the CPA and a decrease in temperature.

ORPL:
Acta Vet. Scand., vol. 32, No. 4, issued 1991, C.O. Bwanga, "Cryopreservation of Boar Sperm", pp. 431-453.

WEST

Generate Collection

L13: Entry 9 of 20 File: USPT Jul 7, 1998

DOCUMENT-IDENTIFIER: US 5776769 A
TITLE: Cell-type specific methods and devices for the low temperature preservation of the cells of an animal species

DEPR:
TL-HEPES medium with 2M glycerol was either one-step or stepwise added to an equal volume of the isotonic sperm suspension to achieve a final 1M glycerol concentration at 22.degree. C. Glycerol in the sperm was removed by dilution either by a one-step or stepwise addition of the TL-HEPES medium with or without an osmotic buffer (sucrose) placed into the cell suspension. The detailed procedures for the glycerol addition and removal are described in Tables 2-5. Sperm motility before, during, and after the different glycerol addition and removal procedures was measured by CASA. The membrane integrity of the sperm was determined by the dual staining technique and flow cytometry.

DEPR:
The addition of CPA to the sperm before cooling and its removal from sperm after warming are two of very important procedures in sperm cryopreservation. The present invention offers a new methodology to define optimal procedures to carry out either or both of these procedures in a fashion to reduce osmotic injury of the sperm. The calculated procedures were implemented and the results agree remarkably well with prediction.

DEPR:
Finally, it should be remembered that the effect of potential chemical toxicity of CPA on sperm cell viability is another reason causing sperm cell injury during the addition and removal of CPA for sperm cell cryopreservation. Given a CPA type and CPA concentration, it is generally accepted that the potential toxicity of the CPA to the cells is decreased with a decrease of cell exposure time to the CPA and a decrease in temperature.

ORPL:
Acta Vet. Scand., vol. 32, No. 4, issued 1991, C.O. Bwanga, "Cryopreservation of Boar Sperm", pp. 431-453.